## RESEARCH COMMUNICATION

# Cathepsin B: an alternative protease for the generation of an aggrecan 'metalloproteinase' cleavage neoepitope

John S. MORT*[1], Marie-Claude MAGNY* and Eunice R. LEE†

*Joint Diseases Laboratory, Shriners Hospital for Children and Department of Surgery, McGill University, 1529 Cedar Avenue, Montreal, Quebec, Canada H3G 1A6, and †Electron Microscopy Unit, Shriners Hospital for Children and Department of Surgery, McGill University, 1529 Cedar Avenue, Montreal, Quebec, Canada H3G 1A6

Previously, only matrix metalloproteinases were believed capable of cleaving the cartilage proteoglycan, aggrecan, between $Asn^{341}$ and $Phe^{342}$, to yield a small G1 fragment terminating in the residues VDIPEN. We show that the combined endo- and exopeptidase activities of the cysteine protease, cathepsin B, also generate this epitope, suggesting that it should no longer be considered as an exclusive marker of metalloproteinase activity.

## INTRODUCTION

Aggrecan, the large aggregating proteoglycan present in articular cartilage, is a multidomain glycoprotein composed of a 220 kDa protein core that is substituted with at least 100 chondroitin sulphate and 30 keratan sulphate chains. Its N-terminal region consists of two globular domains, termed G1 and G2, which are connected by an extended polypeptide interglobular domain (IGD). Whereas no clear function has been assigned to the G2 domain, the G1 domain, together with the small similar glycoprotein, link protein, is responsible for the association of aggrecan with hyaluronate to form immense proteoglycan aggregates. The large size and extremely hydrophilic nature of these assemblies, when restrained within the cartilage collagen network, make this tissue resistant to compression [1].

Loss of proteoglycan from articular cartilage, due to proteolytic cleavage, is thought to be one of the first steps in the degradation of this tissue in arthritis [2]. Recent work has shown that proteolytic cleavage of aggrecan at sites within the IGD occurs in cartilage under normal and pathological conditions. Studies *in vitro* have demonstrated that different proteases cleave the IGD at specific locations [3]. Anti-neoepitope antibodies reacting with the new N- and C-terminals of the resulting degradation products have been prepared and used to assess the involvement of these different enzymes in cartilage metabolism. In particular, cleavage between $Asn^{341}$ and $Phe^{342}$, yielding the neoepitopes … VDIPEN and FFGVGG …, has become accepted as evidence for the action of members of the matrix metalloproteinase family such as stromelysin-1 [4–6].

The lysosomal cysteine proteases cathepsins B and L have also been implicated in various extracellular degradative processes [7]. Whereas cathepsin L is a potent endopeptidase, cathepsin B acts both as an endopeptidase and as a carboxypeptidase, primarily as a peptidyldipeptidase. In a previous study, N-terminal sequencing of the cleavage product of a pig G1/G2 preparation by cathepsin B identified a unique cleavage site in the interglobular region located three residues C-terminal to the 'matrix metalloproteinase' site [3] (Figure 1a). Here we report that, following initial cleavage at this site, the exopeptidase activity of cathepsin B mediates subsequent trimming of the G1-containing fragment to yield the … VDIPEN neoepitope, suggesting that the assignment of this cleavage product to the sole action of matrix metalloproteinases should be reconsidered.

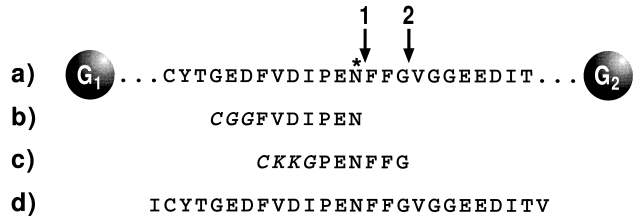


**Figure 1 The interglobular region of aggrecan and the peptides used for production and characterization of anti-neoepitope antibodies**

(**a**) Sequence of the interglobular region between the aggrecan G1 and G2 globular domains, which is susceptible to cleavage by proteases. The sequence is identical for rat, human, pig and mouse. (In bovine aggrecan a serine residue is substituted for the asparagine, indicated by the asterisk.) Arrows indicate the cleavage positions previously described [3] for the matrix metalloproteinases (1) and for cathepsin B (2). (**b**, **c**) Peptides used for production of anti-neoepitope antibodies against the new C-termini produced following cleavage at positions 1 and 2 respectively. Residues in italics denote a spacer segment and the N-terminal Cys added to allow conjugation; (**d**) the extended peptide covering both cleavage sites used to demonstrate antibody specificity.

## MATERIALS AND METHODS

### Materials

Swarm rat chondrosarcoma proteoglycan aggregate was prepared as described previously [8]. Recombinant rat cathepsin B [9] and human cathepsin L [10] were expressed in the yeast *Pichia pastoris* and purified to homogeneity. Peptides were prepared using fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry (FastMoc) on an Applied Biosystems 431A synthesizer and purified by HPLC using an Aquapore C8 column.

Abbreviations used: G1 and G2, first and second globular domains of the aggrecan core protein; IGD, the interglobular domain separating the aggrecan G1 and G2 domains.

[1] To whom correspondence should be addressed (e-mail jmort@shriners.mcgill.ca).

### Antipeptide antibodies

Peptides representing six residues from the C-terminus of the appropriate aggrecan epitope were synthesized with additional N-terminal linkers consisting of a cysteine residue followed by spacer residues. In the case of the … VDIPEN peptide (Figure 1b), two glycine residues were used. However, in the case of the more hydrophobic … PENFFG sequence (Figure 1c), the spacer consisted of KKG for improved solubility. Peptides were coupled to ovalbumin through their terminal cysteine residues using the bifunctional reagent *N*-hydroxysuccinimidyl bromoacetate as described previously [11]. The ovalbumin–CGGFVDIPEN conjugate was used to immunize a rabbit. Extensive characterization of the resulting antiserum and affinity purification of the specific immunoglobulins have been described elsewhere [12,13]. Two mice were immunized with the ovalbumin–CKKGPENFFG conjugate. Initially, 200 $\mu$g of conjugate in PBS was injected intraperitoneally as a 1:1 mixture (200 $\mu$l) with Freund's complete adjuvant. This was followed by two boosts consisting of 100 $\mu$g of conjugate in Freund's incomplete adjuvant (1:1 mixture, 100 $\mu$l) at 3-weekly intervals. The mice were bled out 2 weeks after the last boost. Both animals showed a similar response to the immunizing antigen.

### Aggrecan digestion

Rat proteoglycan aggregate (40 $\mu$g) was incubated with cathepsin B and/or cathepsin L in 50 mM sodium acetate (pH 5.5) containing 0.2 M NaCl, 1 mM EDTA and 10 mM dithiothreitol at 37 °C as described previously by Fosang et al. [3] in a final volume of 35 $\mu$l using an enzyme-to-substrate ratio of 1:100. The reactions were stopped by addition of SDS sample buffer and immediate transfer to a boiling-water bath for 5 min. To study the effect of pH on aggrecan degradation by cathepsin B, the same conditions were used except that 50 mM Mes (pH 4.4–6.5) was used instead of acetate.

### Electrophoresis and immunoblotting

Samples were analysed by SDS/PAGE (10%) under reducing conditions followed by electroblotting to nitrocellulose membranes. Immune localization was as described previously [11]. A 1:200 dilution of mouse serum (anti-PENFFG) or 2 $\mu$g/ml of affinity-purified rabbit anti-VDIPEN were used in the first step, followed by a 1:7500 dilution of alkaline phosphatase-conjugated rabbit anti-mouse or goat anti-rabbit immunoglobulin (Promega, obtained through Fisher Scientific, Montreal, Canada) as the secondary antibody and alkaline phosphatase reaction using Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Antibody specificity was demonstrated by immunoblotting of different peptide–ovalbumin conjugates. For these studies the primary antibody solution (1:200 dilution) contained ovalbumin (1 mg/ml) to suppress reaction with the carrier protein.

## RESULTS

In order to study the products of the aggrecan cleavage by the cysteine proteinases cathepsins B and L, an antipeptide antibody was prepared specific for the neoepitope … PENFFG, which represents the C-terminus of the resulting G1 fragment, inferred from published N-terminal sequencing studies of the products of aggrecan degradation by cathepsin B [3]. This antibody was shown to recognize the immunizing peptide, but not the … VDIPEN peptide or an extended peptide (Figure 1d) covering the region between the last cysteine residue of the G1 domain and nine residues C-terminal to the cathepsin B cleavage site

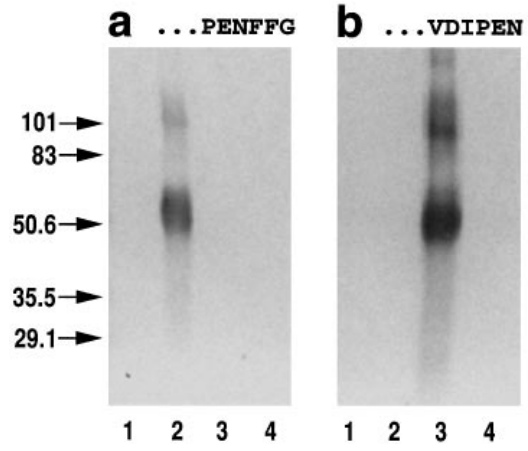


**Figure 2 Characterization of anti-neoepitope antibodies**

Peptide conjugates were analysed by SDS/PAGE and Western blotting using the anti-PENFFG (**a**) and anti-VDIPEN (**b**) antibodies. Lanes 1, ovalbumin (150 ng); lanes 2, ovalbumin–CKKGPENFFG (40 ng); lanes 3, ovalbumin–CGGFVDIPEN (40 ng); lanes 4, ovalbumin–ICYTGEDFVDIPENFFGVGGEEDTV (40 ng). Positions of molecular-mass standards (kDa) are indicated on the left. The upper bands appear to be due to dimerization of the ovalbumin conjugates.

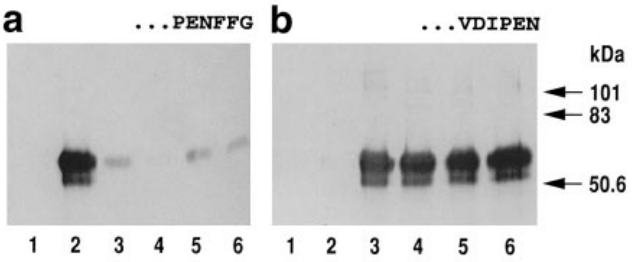


**Figure 3 Cleavage of rat aggrecan IGD by cathepsins B and L**

Aggrecan was digested by cathepsin B and/or cathepsin L at pH 5.5 and the resulting G1 fragment analysed by SDS/PAGE and Western blotting using the anti-PENFFG (**a**) and anti-VDIPEN (**b**) antibodies. Lanes 1, no protease; lanes 2, cathepsin L; lanes 3, cathepsin B; lanes 4, cathepsin B and cathepsin L; lanes 5, cathepsin L followed by cathepsin B; lanes 6, cathepsin B followed by cathepsin L. Positions of molecular-mass standards (kDa) are indicated on the left. Total incubation times were 150 min. In the case of sequential digestions, the second protease was added following 75 min of initial incubation with the first enzyme.

(Figure 2a). Under similar conditions, the … VDIPEN antibody was shown to be specific for its own epitope (Figure 2b).

Rat proteoglycan aggregate was incubated with recombinant cathepsins B or L and the resulting G1 degradation products were analysed by Western blotting using the PENFFG antiserum (Figure 3a). The antiserum revealed two immunostained species in the cathepsin L digests, whereas, in contrast to expectation, very little staining was found in aggregates treated with cathepsin B (Figure 3, lanes 2 and 3 respectively). The immunostained species appeared as a major broad band and as a faster-migrating minor band with the same mobilities as G1 fragments generated on cleavage of rat aggrecan by matrix metalloproteinases, as characterized previously [13] where the two G1 species were shown to be the result of differing degrees of glycosylation.

To test why only a small proportion of the G1 fragments bearing a … PENFFG C-terminus was detected by the antiserum in the cathepsin B digests, rat aggregates were incubated with a

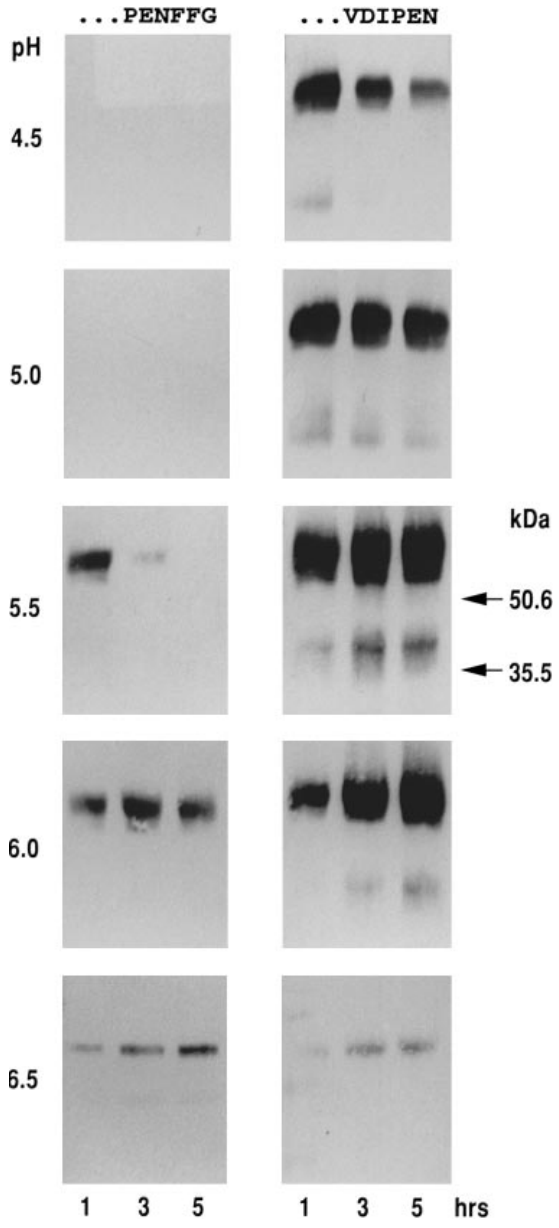


**Figure 4 Effect of pH on aggrecan degradation by cathepsin B**

Rat aggrecan was digested with cathepsin B for 1, 3 or 5 h at the pH values indicated, then analysed by SDS/PAGE and Western blotting using the anti-PENFFG (left column) and anti-VDIPEN (right column) antibodies.

mixture of cathepsin B and L (Figure 3a, lane 4). It was found that when the two cysteine proteinases were added together or when cathepsin B was added after or before cathepsin L (lanes 5 and 6, respectively), the outcomes were identical. In the presence of cathepsin B, almost none of the G1 fragments bearing a ... PENFFG terminus were detected in the blots (Figure 3, compare lane 4 with lane 2). This suggested that all G1 fragments bearing the ... PENFFG C-terminus were further cleaved in the presence of the cathepsin B.

To test whether cleavage of aggrecan by cathepsin B involved the $Asn^{341}$–$Phe^{342}$ bond, proteoglycan aggregate was incubated with the cysteine proteinases and the G1 degradation products were analysed by Western-blotting techniques and the anti-VDIPEN antibody (Figure 3b). In the cathepsin L digests, the blots revealed essentially no G1 fragments terminating in the amino acid residues ... VDIPEN; however, in the mixed digests or in digests treated only with cathepsin B, two intensely stained bands revealed G1 fragments bearing a ... VDIPEN C-terminus (Figure 3, lanes 3–6). Since no processing took place in the presence of cathepsin L alone, it was concluded that under the conditions of testing (pH 5.5) the addition of the cathepsin B rapidly produced new G1 fragments bearing the ... VDIPEN neoepitope.

To study the kinetics of core-protein cleavage by cathepsin B, proteoglycan aggregates were incubated exclusively with cathepsin B at various pH values and for different lengths of time. Blots stained with anti-VDIPEN antibodies or anti-PENFFG antiserum revealed pH-dependent processing of aggrecan by cathepsin B (Figure 4). At pH 6.5, the principal cleavage products were the G1 fragments bearing the PENFFG C-terminus. However, as the pH was reduced (5.5 and below), an essentially complete conversion was observed into G1 fragments bearing the ... VDIPEN C-terminus. In addition to the generation of the two bands described earlier, which represent different glycosylation variants of the G1 domain, a minor lower-molecular-mass band was also observed (Figure 4, pH 5.5), suggesting that cleavage of G1 also occurs within the structure of this domain.

In summary, these studies indicate that following initial endopeptidase cleavage within the IGD, the exopeptidase activity of cathepsin B efficiently removed the tripeptide FFG. Thus it can be concluded that the combined activities of cathepsin B were required to produce the ... VDIPEN neoepitope.

## DISCUSSION

The cysteine proteases cathepsins B and L share with the other members of the papain superfamily a preference for an aromatic or large hydrophobic residue in the $P_2$ position as their principal selectivity determinant [7]. Cleavage of aggrecan IGD between ... FFG and VGG ..., as originally determined by Fosang et al. [3], is completely compatible with this preference. Although there is less rigid selectivity in the $P_1$ position, a glycine residue would be well accepted. Cathepsin L serves as a 'pure' endopeptidase, hence no further processing of the G1 was observed. As noted above, cathepsin B has an additional exopeptidase activity and, particularly at lower pH values, was observed to remove the tripeptide FFG to generate the ... VDIPEN neoepitope. As is clear from the three-dimensional structure of cathepsin B [14], the exopeptidase activity is optimal for the removal of dipeptides (peptidyldipeptidase activity). However, simple, single-residue carboxypeptidase activity of cathepsin B has been known for many years [15] and was observed in the autoprocessing of a cathepsin B hexapeptide C-terminal extension [16]. The trimming reaction could therefore be the result of the removal of a dipeptide followed by a single residue. To date, no cleavages closer to the N-terminus of the IGD than the ... VDIPEN site have been reported, suggesting that the G1 globular domain sterically impedes any further access to this region of the aggrecan molecule.

The sequences of G1 and link protein indicate that these molecules are composed of three subdomains, an N-terminal Ig fold (the subdomain of G1 responsible for protein–protein interaction) followed by a tandem repeat of hyaluronate-binding domains, termed B-loops. While the three-dimensional structure of the aggrecan G1 domain has not yet been determined, an NMR structure for tumour necrosis factor-stimulated gene-6

(TSG-6), another protein containing a B-loop, has been reported [17]. This structure is consistent with the proposal that protease accessibility of the G1 C-terminal region is limited by the globular structure of the protein at the … VDIPEN terminus. However, as shown previously in the case of link protein [18], the Ig fold is somewhat susceptible to proteolysis since fragmented link protein, cleaved within this N-terminally located subdomain, accumulates in human cartilage with age. The gradual appearance of the lower-molecular-mass fragment in the cathepsin B digests of aggrecan suggests that this region is also proteolytically accessible in the G1 domain.

Whereas cathepsin B is active as an endopeptidase over a wide pH range (5–8), the exopeptidase activity, although catalytically much more efficient, is only evident at the lower pH values [19,20]. At the extracellular pH of the cartilage matrix it would be expected that the … PENFFG epitope persists for some time. However, even at elevated pH values, enough exopeptidase activity remains to eventually convert this into the … VDIPEN epitope. Since evidence has been presented for the presence of cathepsin B in the extracellular matrix of articular cartilage [21], this cysteine protease may contribute to the formation of the 'metalloproteinase' G1 neoepitope … VDIPEN and to the degradation of this tissue in arthritis.

We thank Ms. Elisa de Miguel for synthesis and purification of peptides. This work was supported by the Arthritis Society of Canada and the Shriners of North America.

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Received 3 August 1998/1 September 1998; accepted 9 September 1998